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14. ABSTRACT Metastasis to distant vital organs (bone, lung, brain) is the most devastating feature of breast cancer. We proposed to extend our current integrative genomic, proteomic and transcriptomic analysis on the crosstalk between breast cancer cells and bone and lung microenvironments during organ-tropic metastasis. An understanding of secreted metastasis regulators (extracellular proteins, cell-free nucleic acids and small vesicles –exosomes-) has tremendous potential to improve the diagnosis, prognosis and treatment of breast cancer. We hypothesized that <u>tumor and stromal cells communicate via secreted and exosomal proteins and miRNAs to promote organotropic metastasis</u> . Therapeutic disruptions of these communication pathways may significantly increase diagnostic options, improve treatment efficacy and survival of breast cancer patients. The objectives of our proposal are to comprehensively analyze secreted and exosomal proteins and miRNAs that are regulators of bone and lung metastasis, to characterize their function in mediating tumor-stroma interactions, and to determine the potential of utilizing such circulating factors as biomarkers and therapeutic targets. Our specific aims are: 1) Identification and functional characterization of secreted factors promoting bone and lung metastasis; 2) Determination of the role of exosomes in metastatic progression and niche formation; 3) Clinical analysis of metastatic secretome and exosomes.					
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DOD Grant/Contract (Award Number W81XWH-13-1-0427)

YEAR 2 RESEARCH REPORT

Grant Title: Organ-tropic metastatic secretomes and exosomes in breast cancer

INTRODUCTION:

Background: Over 90% of breast cancer deaths are caused by the metastatic spread of tumors to vital secondary organs, including bone and lung. Pathogenesis of metastasis is likely mediated by intercellular communication between tumor cells and the stromal microenvironment. In addition to direct cell-cell contact, many of such tumor-stromal interactions occur via secreted factors, such as growth factors, cytokines, cell-free nucleic acids and small vesicles called exosomes. A comprehensive understanding of secreted molecular mediators of tumor-stroma interactions in organ-tropic metastasis of breast cancer to bone and lung has tremendous potential impact on improving the diagnosis, prognosis and treatment of breast cancer. We postulated that tumor and stromal cells communicate via secreted and exosomal proteins and miRNAs to promote organotropic metastasis. Therapeutic disruptions of these pathways may significantly improve disease diagnosis and prognosis, as well as reducing the morbidity and mortality associated with metastasis. Recently, collaborations between the Lyden and Kang laboratories have demonstrated that exosomes are one of the tumour-derived factors inducing vascular leakiness, inflammation, and BM progenitor cell recruitment during pre-metastatic niche formation and metastasis (Peinado *et al*, Nature Medicine, 2012). The functional biomolecules (*i.e.*, proteins, lipids, RNAs, DNA) contained by exosomes can be horizontally transferred to recipient cells. We showed that an “exosomal protein signature” could identify melanoma patients at risk for metastasis to non-specific distant sites. Moreover, in the context of pancreatic cancer exosomes, we defined the sequential steps involved in liver pre-metastatic niche induction (Costa-Silva *et al*, Nature Cell Biology, 2015). The **objectives** of our proposal are to comprehensively identify secreted and exosomal proteins and miRNAs that are functional mediators of bone and lung metastasis, to characterize their functional mechanisms in mediating tumor-stromal interactions, and to determine the potential of utilizing such circulating factors as biomarkers and therapeutic targets.

Summary of the tasks/aims proposed and achievements:

Task 1: Identification and functional characterization of secreted factors promoting bone and lung metastasis (Months 1-36).

Given the paucity of studies on secreted proteins and miRNAs with functional relevance in metastatic organ-tropism we are currently analyzing secretomes and extracellular miRNAs from lung (**Lyden laboratory**) and bone metastatic breast cancer cells (**Kang Laboratory**).

Task 1a: Identify differentially secreted miRNAs associated with bone-tropism of breast cancer cells (**Months 1-36**). **Dr. Kang's group is responsible for this task.**

Task 1b: Identify differentially secreted proteins and miRNAs associated with lung-tropism of breast cancer cells (**Months 1-36**).

- RNA isolation from lung metastatic cancer cells has been optimized and we are currently performing RNA-Seq at the WCMC Genomics Facility. The data will be deconvoluted and will undergo bioinformatic analysis at the WCMC Institute for Computational Biology, under the supervision of Dr. Olivier Elemento. We used the parental MDA-MB-231 as well as sublines with high and low lung metastatic tropism and the MCFC1h (poorly metastatic) and MCFC1a (highly metastatic) pair of human breast cancer cell lines. We also plan to isolate miRNAs from the 4T1 series of mouse mammary tumor cell lines with progressively higher lung metastatic abilities and are planning on sequencing these (**Months 1-6 of year 3**).

- Once top lung tropic secretome miRNA and proteins are identified, we plan to test the detection of secretome candidate proteins/miRNAs in animal models: healthy, primary tumor bearing, spontaneous and experimental lung metastasis. 40 nude and Balb/c mice will be used (**Months 6-12 of year 3, as soon as ACURO approval is obtained**).

- We are currently working on methods to optimize the comparison between secreted and exosomal miRNA/proteins isolated from lung-tropic and parental control cell lines.

Outcome and Milestones: We have identified distinct protein profiles (**Lyden**) of breast cancer cell lines with differential lung metastatic capabilities whose pathological relevance can be validated in animal models of lung metastasis. We have published a subset of these the results, pertaining to lung exosome protein content, specifically integrins, at the end of the second year of funding, ahead of the milestone timetable. For **Year 3** of funding studies, we will focus on moving further the molecular and functional analysis of lung tropic exosomal miRNA (**Lyden**).

Task 1c: Stroma-derived miRNAs as biomarkers and potential therapeutic targets (**Months 1-24**). **Dr. Kang's group is responsible for this task.**

Task 2: Determination of the role of exosomes in metastatic progression and niche formation (Months 1-48). *Task will be performed by Dr. Kang (bone metastatic exosomes & functional analysis), Dr. Lyden (lung metastatic exosomes & functional analysis), Dr. Garcia (proteomics), Sequencing Core Facilities (RNA-Seq).*

Based on our previous studies, tumor-derived exosomes can promote metastasis by transfer of functional factors. This aim will analyze exosomal proteins and miRNAs released from lung metastatic breast cancer cells to identify metastasis regulators.

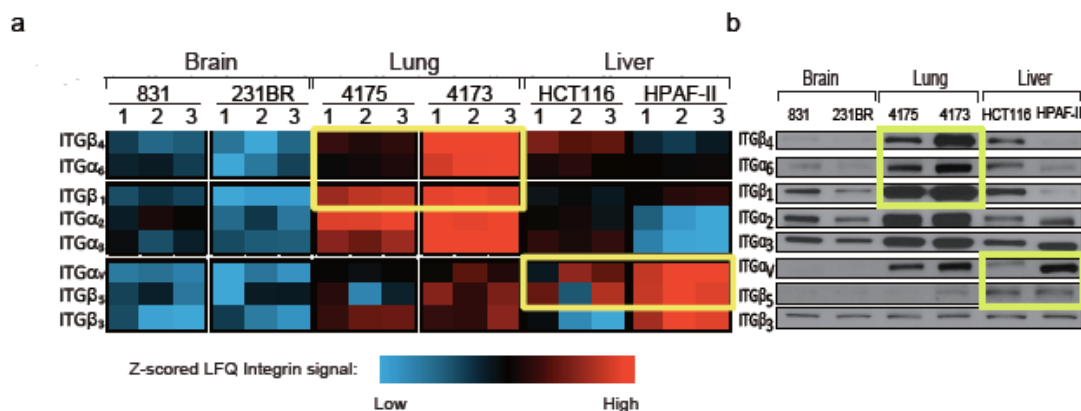
Task 2a: Identify differences in exosomal protein/miRNA composition between highly and poorly bone metastatic breast cancer cells and determine the function of the candidate exosomal bone metastasis regulators (**Months 1-48**). **Dr. Kang's group is responsible for this task.**

Task 2b: Identify differences in exosomal protein/miRNA composition between highly and poorly lung metastatic breast cancer cells and determine the function of the candidate exosomal lung metastasis regulators (**Months 1-48**).

- For the second year of funding we have focused on performing a comprehensive mass spectrometry proteomic analysis of exosomes isolated from various organotropic cell lines, focusing specifically on lung-tropic metastatic cells. We isolated and characterize exosomes from cancer cells with different lung metastatic capabilities, and analyzed protein by mass-spectrometry. We used the parental MDA-MB-231 as well as sublines with high and low lung metastatic tropism. (**Months 1-6 of year 2**).

We postulated that exosomal adhesion molecules could regulate local microenvironments within future metastatic organs. Quantitative mass spectrometry of brain-, lung-, and liver-tropic metastatic exosomes identified six integrins among the top 40 most abundant adhesion molecules, making integrins the most highly represented protein family in this analysis. These data indicate a correlation between exosomal integrins and metastatic tropism (Fig.1)

Figure 1. Integrin expression in human breast cancer and pancreatic metastatic cell line-derived exosomes, based on a) quantitative mass spectrometry analysis and b) Western blot analysis.



Interestingly, we found that integrin expression profiles correlated with tissue organotropism. Both quantitative mass spectrometry (Fig. 1a) and western blot analysis (Fig. 1b) revealed that integrin alpha 6 (ITG α_6), and its partners ITG beta 4 (ITG β_4) and ITG beta 1 (ITG β_1), were present abundantly in lung-tropic exosomes. In contrast, ITG beta 5 (ITG β_5), which associates only with ITG alpha v (ITG α_v), was detected primarily in liver-tropic exosomes (Fig. 1). We confirmed these findings by exosome proteomics on 28 organ-specific metastatic cell lines (Tables 1 and 2). Qualitative mass spectrometry revealed that ITG α_6 was present in lung-tropic exosomes, whereas ITG β_5 was found in liver-tropic exosomes (Tables 1 and 2) consistent with our quantitative proteomics data. Exosomes

from 4173, 4175, and 4180 lung-tropic MDA-MB-231 variants expressed ITG $\alpha_6\beta_4$ (Table 1). Meanwhile, ITG beta 3 (ITG β_3) was present in exosomes isolated from brain tropic cells (Table 1). Notably, unlike non-cancerous lung fibroblast WI-38 or epithelial MCF10A exosomes, metastatic cell exosomes contained ITG $\alpha_2\beta_1$, suggesting that this integrin could serve as a biomarker for metastasis (Table 1). Importantly, exosomal integrin expression does not necessarily reflect cellular integrin expression, consistent with selective packaging of integrins in exosomes (Fig. 2). Taken together, our data suggest that exosomal integrin expression patterns underlie organotropism to the lung, liver, and brain.

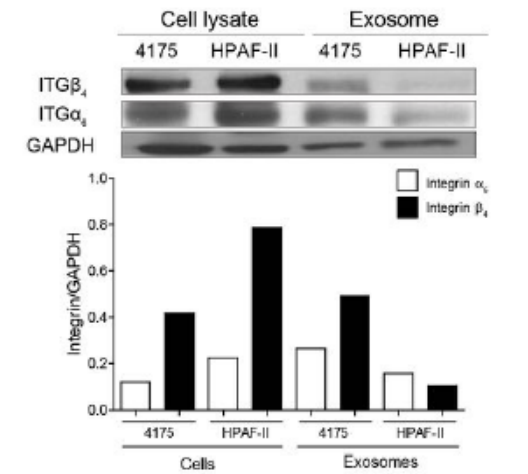
Table 1. Integrin expression in human metastatic cell line-derived exosomes, based on qualitative mass spectrometry analysis.

[illegible]

Table 2. Integrin expression in human versus murine metastatic cell line-derived exosomes, based on qualitative mass spectrometry analysis

Human			Murine		
Sites of metastasis	Majority to lung	Lung and liver	Sites of metastasis	Lung	Liver
Cell type	Breast cancer		Cell type	Breast cancer	Pancreatic cancer
Cell line	MDA-MB-231	MDA-MB-468	Cell line	E0771	Pa02
ITGa ₁			ITGa ₁		
ITGa ₂	+	+	ITGa ₂		
ITGa _{2b}			ITGa _{2b}		+
ITGa ₃	+	+	ITGa ₃	+	+
ITGa ₄			ITGa ₄		
ITGa ₅		+	ITGa ₅	+	+
ITGa ₆	+	+	ITGa ₆	+	+
ITGa ₇		+	ITGa ₇	+	+
ITGβ ₁	+	+	ITGβ ₁	+	+
ITGβ ₂	+	+	ITGβ ₂	+	+
ITGβ ₄		+	ITGβ ₄		+
ITGβ ₅		+	ITGβ ₅		+
ITGβ ₆		+	ITGβ ₆		

Figure 2. Distinct levels of integrin expression in exosomes versus cell line of origin indicates selective packaging of integrins in exosomes.



We then sought to determine downstream effects of exosomal interaction with target cells, and thus educated Kupffer cells *in vitro* with either BxPC-3 or BxPC-3 ITGβ₅KD exosomes every other day for two weeks. Unbiased analysis of gene expression by RNA sequencing in Kupffer cells identified 906 genes upregulated over two-fold following treatment with BxPC-3 exosomes compared to BxPC-3 ITGβ₅KD exosomes. Cell migration genes were most prominently

upregulated (two-fold for 221 genes; four-fold for 42 genes).

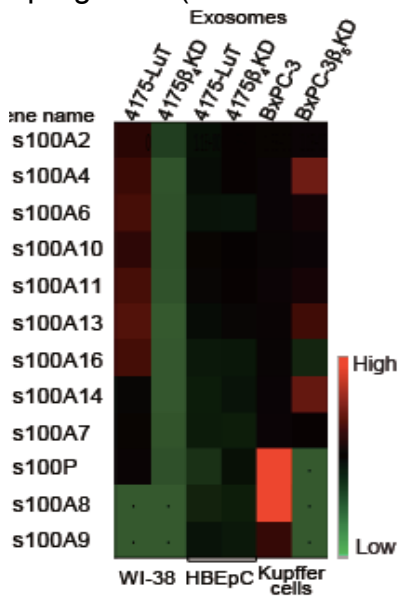


Figure 3. S100 gene induction in lung and liver stromal cells upon lung and liver –tropic exosome treatment, respectively.

Of these, *S100A8*, *S100A9* and *S100P* were upregulated over four-fold (Fig.3; GEO accession number GSE68919). Since pro-inflammatory *S100* gene expression correlates with metastasis, we analysed *S100* genes in tumour exosome-educated lung WI-38 fibroblasts and human bronchial epithelial cells (HBEpCs). Multiple *S100* genes (*S100A4*, -*A6*, -*A10*, -*A11*, -*A13*, and -*A16*) were upregulated more than five-fold upon WI-38 fibroblast treatment with 4175-LuT exosomes *versus* 4175-LuT ITGβ₄KD exosomes (Fig. 3). Interestingly, *S100* genes remained unchanged in HBEpCs treated with 4175-LuT exosomes (Fig. 3). Moreover, exosome-treated lung fibroblasts proliferated and migrated more compared to controls. We then surveyed, by in-cell western blot analysis, ITGβ₄-signaling proteins in WI-38 fibroblasts treated with 4175-LuT- or 4175β₄KD exosomes. Notably, only Src/p-Src levels increased in an exosomal ITGβ₄-dependent manner (Fig. 4), consistent with the known roles of ITGα₆β₄ in Src activation and *S100A4* expression. Therefore, in addition to their adhesive properties, exosomal integrins can activate Src and upregulate pro-migratory and pro-inflammatory *S100* molecules in specific resident cells within distant tissue microenvironments, influencing the expression of genes implicated in facilitating tumour metastasis.

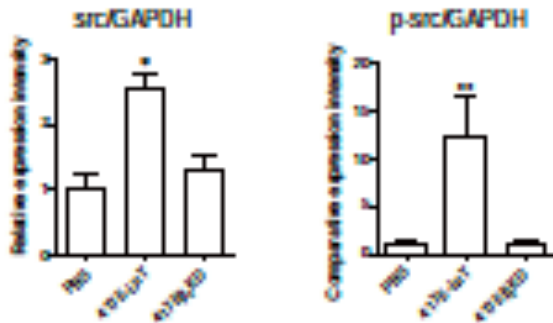


Figure 4. Exosomal integrin-dependent induction of pSrc in lung stromal cells.

FUTURE DIRECTIONS FOR YEAR 3:

- We plan to test the detection of exosomal proteins/miRNAs in plasma of animal models: healthy, primary tumor bearing, and bone metastases bearing mice (**Months 24-36, as soon as ACURO approval is obtained**)).
- We will create KD/OE lines of candidates and evaluate their metastatic capabilities. We will use exosomes from generated cell lines to treat mice bearing primary tumors and bone metastases, and evaluate their effects (**Months 24-36**).
- We will label exosomes with dyes or epitope tags and investigate the cell identity of target cells. We will then characterize the downstream effects induced *in vivo* in target cells following exosome uptake (**Months 24-36**).

Task 2c: Investigate the functional role of stroma-derived exosomes in metastasis (**Months 24-36**). **Dr. Kang's group is responsible for this task.**

Outcome and Milestones: We have identified several candidate exosomal proteins and miRNAs from tumor and associated stromal cells whose manipulation changes the metastatic abilities of breast cancer cells. We expect to identify target cells and downstream effects of exosome uptake. Multiple papers reporting the role of exosomes in bone and lung metastasis will be published in years 3 and 4.

Upon receipt of ACURO approval for animal studies, we will focus on a detailed analysis of the *in vivo* biodistribution of exosomes isolated from organ-tropic cell lines. Moreover, we will combine exosome labeling with immunofluorescence studies using cell-type specific markers to identify the specific cells uptaking tumor exosomes in each destination organ. We will perform these approaches *in vivo* during the second year of funding.

Task 3: Clinical analysis of metastatic secretome and exosomes (Months 24-60). In collaboration with Dr. Bromberg (clinical sample collection and analyses) we have been accruing and isolating exosomes from plasma samples from breast cancer patients with metastasis to various sites (lung, brain, bone). Based on our proteomics analysis of metastatic breast cancer cell lines, we identified exosomal proteins, specifically integrins, functionally relevant in organ-tropic, specifically lung-tropic breast cancer metastasis, and tested these

samples by ELISA (protein). We predicted that due to their extracellular localization, secreted factors represent superior biomarkers and therapeutic targets.

Therefore, guided by the results of our exosomal proteomics analysis, we performed ELISA assays for plasma-derived exosomal integrins in patients with lung or liver metastasis (ITG β_4 or ITG α_v , the binding partner of ITG β_5 , respectively). We found elevated ITG β_4 levels in exosomes from patients with lung metastasis (regardless of tumor-type) *versus* patients with no metastasis or liver metastasis (Fig. 6). Exosomes isolated prior to metastasis expressed high exosomal ITG β_4 , successfully predicting which breast cancer patients would progress to develop lung metastasis (POD) (Fig. 5a). ITG α_v was significantly increased in exosomes isolated from cancer patients with liver metastasis *versus* patients with no metastasis or lung metastasis (Fig. 6). Finally, exosomal ITG α_v levels at diagnosis were higher in pancreatic cancer patients who developed liver metastasis compared to those without liver metastasis within three years post-diagnosis and to control subjects (Fig. 5b). Taken together, our data indicate that the specific exosomal integrins in breast and pancreatic cancer patient plasma correlate with and predict likely sites of metastasis.

Figure 5. Exosomal integrin expression as a potential predictor of patient organ metastasis.
a, Exosomal ITG β_4 levels in breast cancer patients who were metastasis-free at the time of blood draw. **b**, Exosomal ITG α_v in pancreatic cancer patients metastasis-free at the time of blood draw.

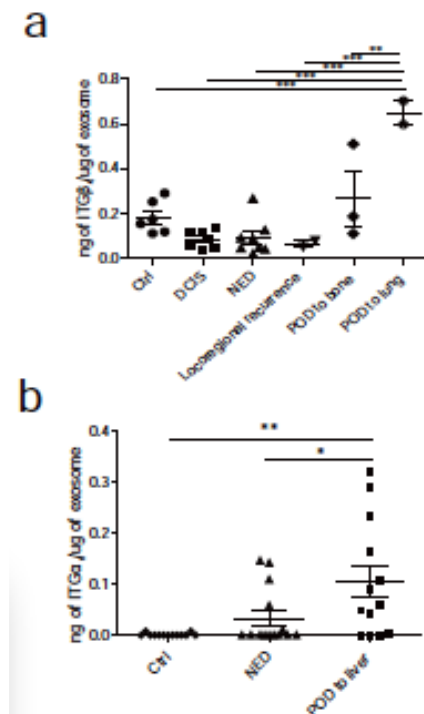
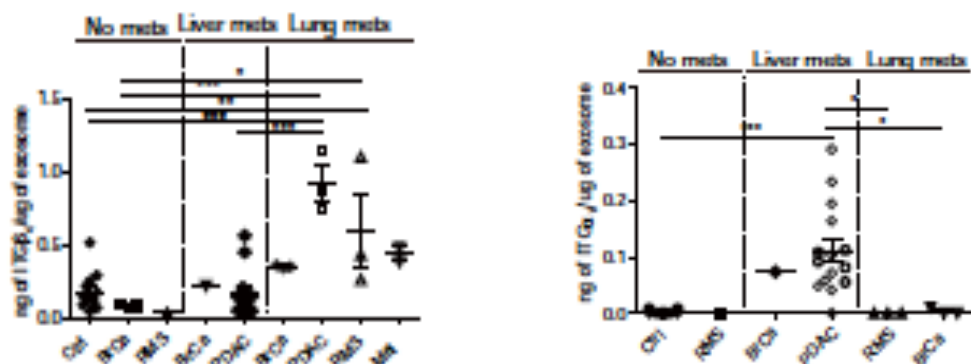


Figure 6. Exosomal integrin expression as a potential metastatic site biomarker in healthy subjects versus patients with breast cancer, pancreatic cancer, rhabdomyosarcoma and melanoma.



Outcome and Milestones: We expect to validate candidates identified in Tasks 1 and 2 in a larger cohort of patient samples. We expect that some factors will have diagnostic and/or predictive value. Those with function in metastasis may become potential therapeutic targets. These results will be published in several papers in year 5.

KEY RESEARCH ACCOMPLISHMENTS:

Publications:

1) Tumour exosome integrins determine organotropic metastasis.

Hoshino A, Costa-Silva B, Shen TL, Rodrigues G, Hashimoto A, Tesic Mark M, Molina H, Kohsaka S, Di Giannatale A, Ceder S, Singh S, Williams C, Soplop N, Uryu K, Pharmed L, King T, Bojmar L, Davies AE, Ararso Y, Zhang T, Zhang H, Hernandez J, Weiss JM, Dumont-Cole VD, Kramer K, Wexler LH, Narendran A, Schwartz GK, Healey JH, Sandstrom P, Jørgen Labori K, Kure EH, Grandgenett PM, Hollingsworth MA, de Sousa M, Kaur S, Jain M, Mallya K, Batra SK, Jarnagin WR, Brady MS, Fodstad O, Muller V, Pantel K, Minn AJ, Bissell MJ, Garcia BA, Kang Y, Rajasekhar VK, Ghajar CM, Matei I, Peinado H, Bromberg J, Lyden D. **Nature**. 2015 Nov 19;527(7578):329-35. doi: 10.1038/nature15756. Epub 2015 Oct 28. PMID:26524530

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Co-Organizer/Host – CNIO Frontiers Meeting, Madrid, Spain, September 2015 - Co-host with Editors from Cancer Cell, Nature Medicine, and Nature Reviews Cancer and Dr. Hector Peinado

Metastasis Initiation: Mechanistic and Therapeutic Opportunities”

Spanish National Cancer Research Labs (CNIO)

Dr. Lyden has presented preliminary data from these studies in 10 national and international meetings this past year. The top 3 meetings were:

- **Keynote Speaker and the Mina J. Bissell Awardee**

Institute of Molecular Pathology and Immunology

University of Porto 25th anniversary celebration

Porto, Portugal, March 2015

- **Invited Speaker**

Biology of Cancer: Microenvironment, metastasis, and the therapeutics

Cold Spring Harbor Laboratory

Cold Spring Harbor, NY, May 2015

- **Keynote Address**

Tumor Metastasis AACR Meeting

November 30 - Dec 3, 2015

Hyatt Regency Austin

Austin, Texas, USA

CONCLUSION:

We have defined a specific repertoire of integrins expressed on tumour-derived exosomes, distinct from tumor cells, which dictates exosome adhesion to specific cell-types and ECM molecules in particular organs. Importantly, exosomes expressing ITG $\alpha_v\beta_5$ specifically bind to Kupffer cells, mediating liver tropism, whereas exosomal ITG $\alpha_6\beta_4$ and ITG $\alpha_6\beta_1$ bind lung-resident fibroblasts and epithelial cells, governing lung tropism. The biological data confirm that highly malignant, organ-tropic breast cancer cell lines secrete exosomes with increased amount of protein and can be transferred to specific types of stromal cells, depending on the tropism of the cancer cell line the exosomes were derived from.

Cell type-specific exosome integrin uptake promoted pro-migratory and pro-inflammatory *S100* gene upregulation (*S100A4*, -A6, -A10, -A11, -A13, and -A16 in lung fibroblasts; *S100P* and -A8 in Kupffer cells). Notably, tumour exosomes failed to elicit *S100* upregulation in lung epithelial cells, highlighting the cell-type specificity of exosomal education. Exosomal integrins not only promote adhesion, but also trigger signalling pathways and inflammatory responses in target cells resulting in the education of that organ rendering it permissive for the growth of metastatic cells.

The identification of molecules expressed on exosomes that could “address” exosomes to specific metastatic sites could predict metastatic niches and allow foreseeing metastatic spread of tumors and metastatic organ.

We propose to test this hypothesis during next year in animal models of organ-tropic breast cancer metastasis.

Impact: Our research will unveil novel secreted and exosomal proteins and miRNAs as functional regulators of long-range communications between metastatic tumor and stromal microenvironment. Moreover, due to their extracellular localization, secreted factors represent superior biomarkers and therapeutic targets as they can readily enter body fluids where they can be non-invasively detected, targeted or restored. Thus, we expect this research to open up exciting novel avenues of clinical translation in early breast cancer and metastasis detection, prognosis and therapy. Therapeutic strategies may include, but not be limited to, monoclonal antibody for integrin, S100 or Src targeting, restoration of metastasis-inhibiting miRNAs and proteins, and modulation of signaling pathways activated by secreted proteins or targeted by miRNAs.

REFERENCES: Not applicable. No references are associated with this report.

APPENDICES: Not applicable. No appendices are attached to this report.

SUPPORTING DATA: Figures 1-6, Tables 1-2